

# Screening of Mandarin Oil on Indomethacin Induced Inflammatory Bowel Disease in Wistar Rats

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## ABSTRACT

**Objective:** The present study investigates the protective effect of Mandarin Oil against Indomethacin (INDO) induced Inflammatory Bowel Disease (IBD) in male Wistar rats. **Material and Method:** IBD was induced by administration of indomethacin solution (7.5 mg/kg b.w., s.c) for 2 days. The study comprises of 5 groups (n=6), normal saline treated, disease induced, Mandarin Oil-I (200 mg/kg), Mandarin Oil-II (400 mg/kg) and Sulfasalazine (standard drug, 500 mg/kg b.w, p.o) treated group. After treatment period animals were sacrificed by cervical dislocation and dissected. Ileum was isolated for macroscopic, microscopic and biochemical studies. **Results:** Pre-treatment with Mandarin oil shows decrease in macroscopic score for inflammation as compared to indomethacin treated group. Histopathological examination of Mandarin oil treated group revealed less damage compared to indomethacin treated group. **Conclusion:** Mandarin oil have shown to be effective in indomethacin-induced enterocolitis in rats, which has protected the animals against experimentally induced disease because of its antioxidant and anti-inflammatory activities.

**Key words:** mandarin oil, inflammatory bowel disease, indomethacin, sulfasalazine and anti-oxidant.

## INTRODUCTION

Crohn's disease and ulcerative colitis are two idiopathic inflammatory bowel disorders. Inflammatory bowel disease is now thought to represent an inappropriate response of the intestinal mucosal immune system which leads to upregulation of the synthesis and release of different pro inflammatory mediators, including eicosanoids, platelet activating factor, cytokines and reactive oxygen and nitrogen metabolites.<sup>1</sup> The infiltrated and activated neutrophils represent an important source of reactive oxygen and nitrogen species. Free radicals, neutrophils can also release proteases, lactoferrin and lipid mediators that can contribute to intestinal injury. Macrophages produce certain cytokines, such as tumor necrosis factor (TNF- $\alpha$ ) and interleukin, the levels of which are often increased in both animal models and patients with ulcerative colitis.<sup>2</sup> In many studies, it has been reported that antioxidants show beneficial effects on

experimental colitis.<sup>3</sup> It has been suggested that the imbalance between pro-oxidant and antioxidant mechanisms in IBD may be controlled by antioxidant treatment.<sup>4</sup> Mandarin oil possess antioxidant property and this situation, requires an external supply of antioxidants which is necessary to regain a balance between free radicals and antioxidants.<sup>5</sup> Traditionally oil is used as antidyspeptic, antiscorbutic, stomachic, astringent, carminative, tonic, antiulcer, colic, anthelmintic, antidiarrhoeal, appetizer, and in spasmodic affection, helminthiasis, bilious diarrhoea & dyspepsia.<sup>6</sup> The chemical constituents viz. limonoids, flavonoids (flavonone glycoside), phenolic acid are present in the Mandarin oil. The peel of citrus fruits is a rich source of flavonone and many polymethoxylated flavones, which are very rare in other plants.<sup>7</sup>

Previously Mandarin oil has shown antioxidant, antifungal, antimicrobial, diabetic,

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hepatoprotective, antianxiety and anticancer properties.<sup>8-13</sup>

The present study is to investigate the effects of Mandarin oil on the experimental model of colitis induced by trinitrobenzenesulphonic acid (TNBS) in wistar rats, it is well established model of inflammatory bowel disease that has same histological and biochemical features of human disease.

## MATERIALS AND METHODS

### Animals

Healthy male Wistar rats weighing 200-250 g were used in the study. Animals were harbored on a 12-h light/dark cycle at a constant ambient temperature ( $24 \pm 1^\circ\text{C}$ ) with normal rat chow and water available *ad libitum*. The study was reviewed and approved by the Institutional Animal Ethical Committee.

### Acute Oral Toxicity Study

The acute oral toxicity was carried out as per Guidelines set by OECD, revised draft guidelines 423. The Mandarin oil sample did not exhibit any toxicity symptoms and mortality when given orally at dose of 2000 mg/kg body weight. Hence 1/10<sup>th</sup> and 1/5<sup>th</sup> i.e. 200 mg/kg and 400 mg/kg b.w. were selected for the study.

### Indomethacin (INDO) Induced Enterocolitis in rats<sup>14</sup>

30 animals were randomly divided into five groups, each consisting of 6 animals as follows. Group 1 served as a normal control and maintain on regular rat food and drinking water *ad libitum*. All remaining Groups received 7.5 mg/kg indomethacin (solubilized in 100% alcohol then diluted with 5% w/v sodium bicarbonate solution). Group 2 animals received indomethacin without any treatment. Group 3 and 4 are pre-treated with Mandarin oil in dose of 200 & 400 mg/kg, prior to administration of indomethacin on 08<sup>th</sup> and 09<sup>th</sup> day. Mandarin oil was continued till 11<sup>th</sup> day. Group 5 served as standard drug treated group, which received indomethacin and treated with sulfasalazine which was suspended in distilled water using 0.5% w/v CMC solution and administered orally.

### Assessment parameters

#### Feed Intake

Weighted amount of feed was given to the rat every day. The feed intake was calculated by subtracting the remaining feed with the given feed weight.

### Macroscopic Scoring<sup>15</sup>

Blood was collected through retro-orbital puncture. Following the blood collection, the animals were sacrificed and the small intestine (ileum segment) were excised and opened longitudinally. Gross findings were ranked using the following criteria, 0 - No change in both serosa and mucosa, 1-hyperemia and petechial, bleeding, 2-single mucosal erosion or ulceration, 3-single mucosal erosion or ulcer with hyperemic, adhesive or hemorrhagic lesions in the serosa and mesentery, 4-multiple erosions or ulcerations (less than 10 cm), 5-Multiple erosions or ulcerations (more than 10 cm)

### Biochemical Assays

#### Serum Lactate Dehydrogenase (LDH)<sup>16</sup>

The serum was used for determination of LDH, indicator of tissue damage by using commercially available diagnostic kit (ERBA Diagnostics, Mannheim GmbH, Germany).

#### Myeloperoxidase (MPO) Assay<sup>17</sup>

Pieces of inflamed tissues (rat colon - 2 cm / rat ileum - 2 cm) were taken. The tissue was then rinsed with ice-cold saline, blotted dry, weighed and excised. Minced tissue was homogenized in 10 volumes of ice-cold potassium phosphate buffer (pH 7.4) using tissue homogenizer. The homogenate was centrifuged at 3500 rpm for 30 min at 4°C. The supernatant was discarded, 10 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (HETAB) and 10 mM EDTA was then added to the pellet. It was then subjected to one cycle of freezing and thawing for brief period (15 sec) of sonication. After sonication solution was centrifuged at 15,000 rpm for 20 min. Myeloperoxidase (MPO) activity was measured spectrophotometrically as follows: 0.1 ml of supernatant was combined with 2.9 ml of 50mM phosphate buffer containing 0.167 mg/ml O-dianisidine hydrochloride and 0.0005% H<sub>2</sub>O<sub>2</sub>. The change in absorbance was measured spectrophotometrically, at 460 nm.

#### Calculation of MPO activity

$$\text{MPO activity} = \frac{X}{\text{wt of the peice of the tissue taken}}$$

$$\text{Where; } X = \frac{10 \times \text{Change in Absorbance per Minute}}{\text{Vol of Supernatant taken in final rean}}$$

#### Colonic glutathione content: (GSH)<sup>16</sup>

Briefly, GSH in tissue homogenate was reacted with Ellman's reagent (5, 5-dithio-2-nitrobenzoic acid) in phosphate buffer saline (PBS, pH 8.0) and the absorbance was measured at 412 nm.

## Assay Procedure

Reagents	Blank	Test
Ellman's reagent	50 $\mu$ l	50 $\mu$ l
Reaction buffer	2.5 ml	2.5 ml
Tissue homogenate	-	250 $\mu$ l

Mix well and incubated at room temperature for 15 minute Absorbance measured at 412 nm on UV spectrophotometer.

## Calculation

$$GSH \text{ Mole} = \frac{\text{Absorbance}}{1.4550 \times 10^4} \times 11.2 \text{ Mole}$$

## Colonic lipid peroxides concentration (LPO)<sup>16</sup>

Lipid peroxidation, an indicator of mucosal injury induced by reactive oxygen species was measured as thiobarbituric acid reactive substance. Briefly, 0.5 ml of colonic tissue homogenates prepared were reacted with 2 ml of TBA reagent containing 0.375% TBA, 15% trichloroacetic acid and 0.25 N HCl. Samples were boiled for 15 min, cooled and centrifuged. Absorbance of the supernatants was measured by spectrophotometer measured at 532 nm.

## Calculation

$$\text{Concentration } (\mu\text{M}) = \frac{\text{Absorbance}}{\text{Molar extinction coefficient}} \times 10^6$$

Final Concentration of unknown sample/gram tissue =  $100 \times \mu\text{M LPO equivalent/gram tissue}$ .

## Histopathological Studies<sup>18</sup>

A portion of inflamed ileum was fixed in phosphate-buffered formaldehyde and embedded in paraffin and 5 $\mu$ m sections were prepared. Tissues were stained with hematoxylin and eosin and were evaluated by light microscopy.

## Statistical Analysis

Results were expressed as Mean  $\pm$  SEM. The statistical significance of any difference in each parameter among the groups was evaluated by one-way ANOVA, using Dunnet's t test as *post hoc* test.

## RESULTS

### Indomethacin Induced Enterocolitis in Rats

#### Feed Intake and Macroscopic Score

There was severe anorexia in indomethacin treated group with decrease in average feed intake ( $P < 0.01$ ) as shown in (Figure: 1). Macroscopic evidence of extensive colonic mucosal injury was assessed by the macroscopic score (Figure: 2). MO-I treated group showed a significant ( $P < 0.001$ ) decrease in macroscopic score, whereas less significant ( $P < 0.05$ ) decrease was observed with MO-II treatment as compared to that of indomethacin treated group.

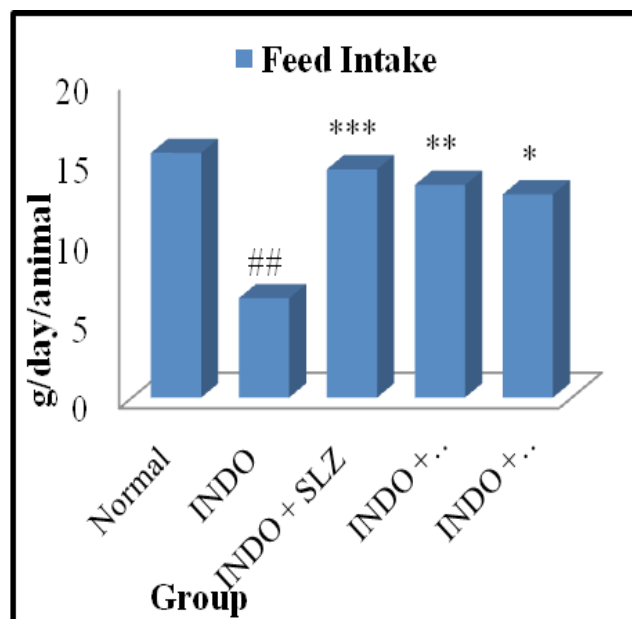


Figure 1: Effect of MO on Feed intake

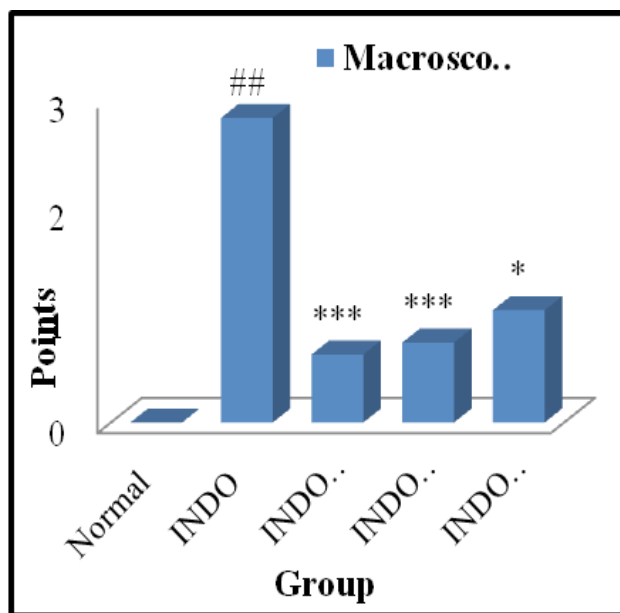


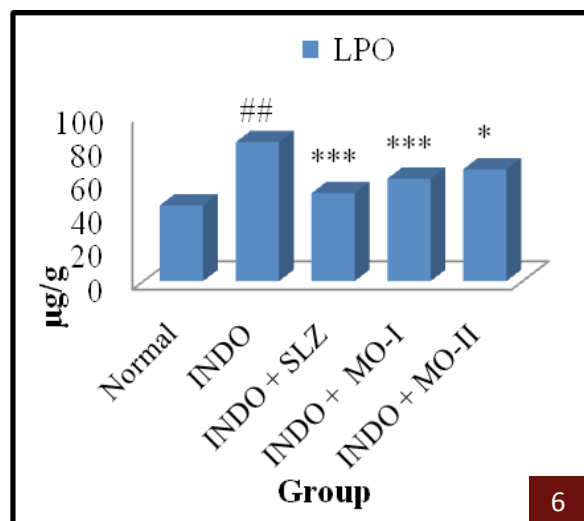
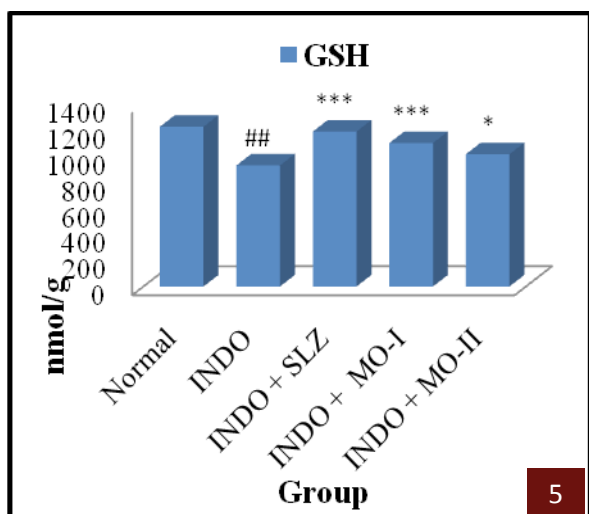
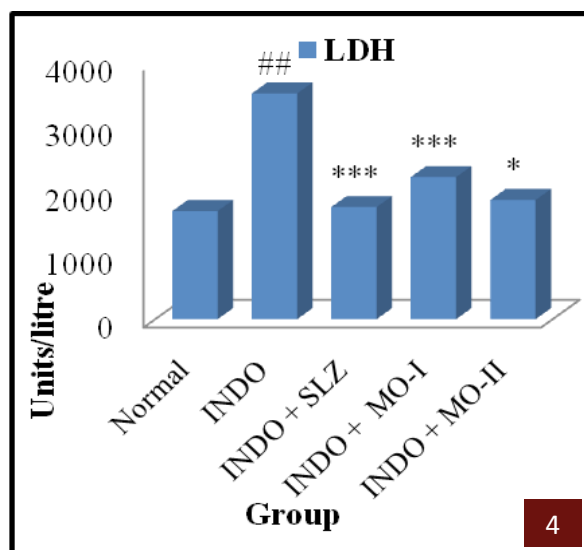
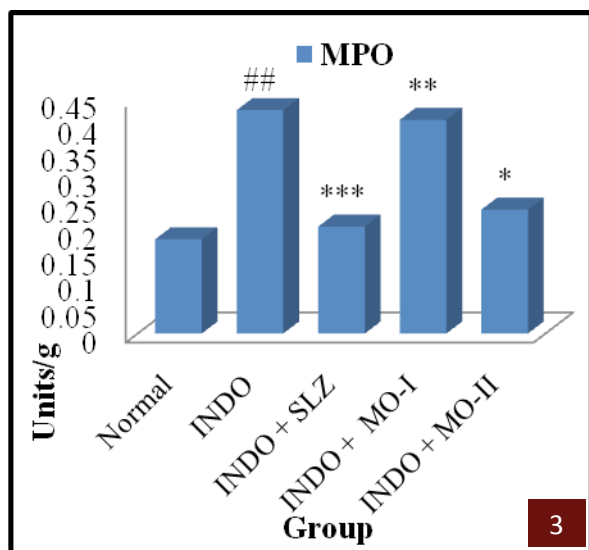
Figure 2: Effect of MO on Macroscopic score in Indomethacin induced enterocolitis in rats.

## $P < 0.01$  when compared to Control, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  when compared with Indomethacin.

**Table 1: Effect of treatment on MPO, LDH, GSH, LPO, Feed intake and Macroscopic scoring**

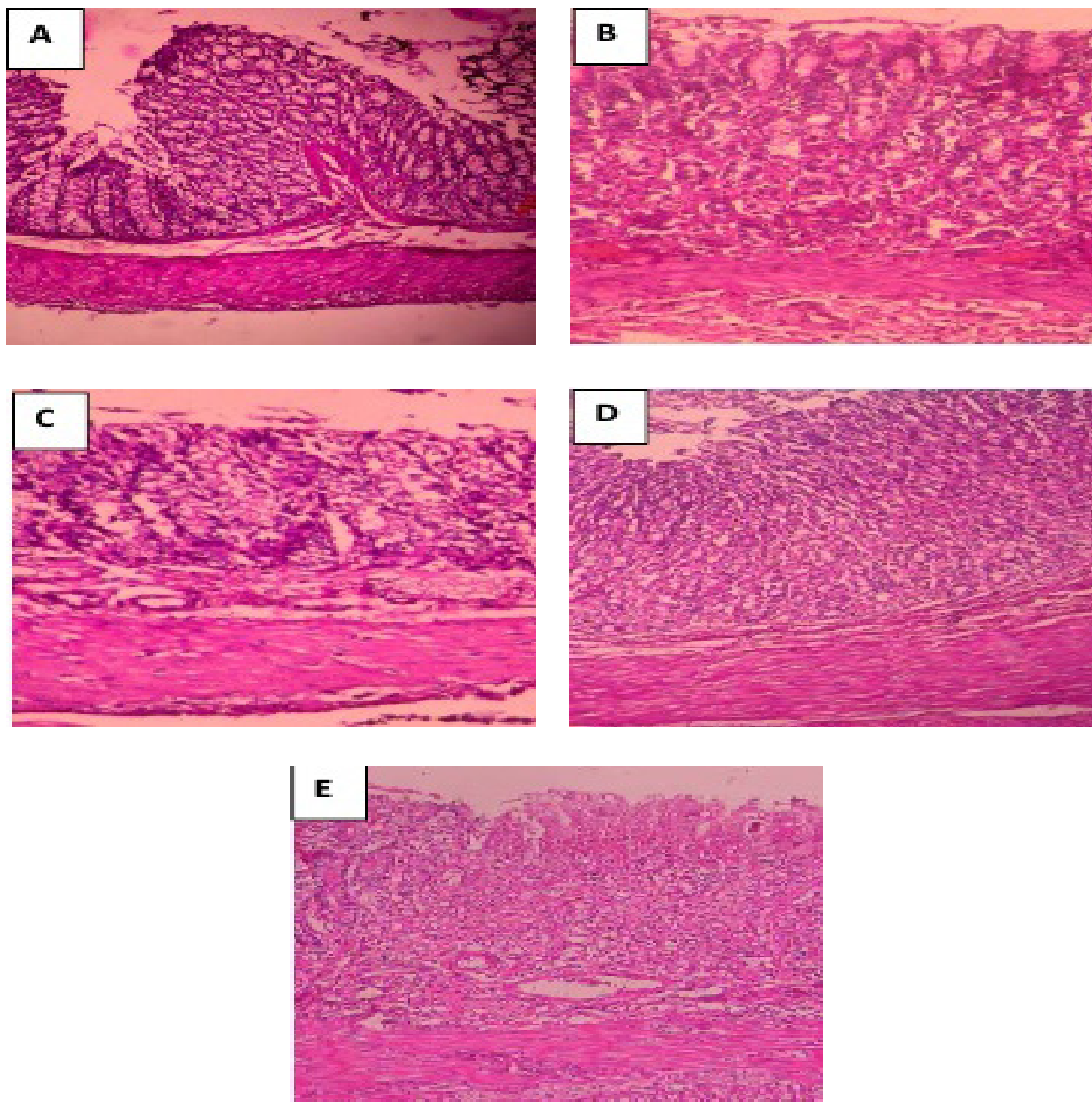
Group	MPO activity (units/g)	Serum LDH (units/litre)	Colonic GSH concentration (nmol/g of wet tissue)	Colonic LPO concentration (µg/g of wet tissue)	Feed intake (g/day/animals)	Macroscopic score (points)
Normal	0.1805±0.001	1688 ±13.713	1226±3.138	45.41±1.11	15.41±0.184	0.0
INDO Treatment	0.4278±0.002 <sup>##</sup>	3525±16.967 <sup>##</sup>	929±2.186 <sup>##</sup>	83.23±0.89 <sup>##</sup>	6.26±0.1206 <sup>##</sup>	2.82±0.02 <sup>##</sup>
INDO+ SLZ	0.2048±0.001 <sup>***</sup>	1751±11.406 <sup>***</sup>	1189±1.701 <sup>***</sup>	52.67±1.30 <sup>***</sup>	14.38±0.07 <sup>***</sup>	0.63±0.01 <sup>***</sup>
INDO + MO-I	0.4092±0.006 <sup>**</sup>	2219±23.56 <sup>***</sup>	1101±7.346 <sup>***</sup>	61.32±0.45 <sup>***</sup>	13.39±0.10 <sup>**</sup>	0.74±0.01 <sup>***</sup>
INDO + MO-II	0.2372±0.001 <sup>*</sup>	1861±13.37 <sup>*</sup>	1015±1.317 <sup>*</sup>	66.99±0.48 <sup>*</sup>	12.80± 0.05 <sup>*</sup>	1.04±0.21 <sup>*</sup>

Values are expressed as mean ± S.E.M for 6 animals in each group; One Way ANOVA followed by Dunnett's Test : <sup>##</sup>P<0.01 when compared to Control, <sup>\*</sup>P< 0.05, <sup>\*\*</sup>P<0.01, <sup>\*\*\*</sup>P<0.001 when compared with Indomethacin



**Effect of MO-on bio-chemical enzyme, MPO (Figure 3), LDH (Figure 4), GSH (Figure 5) and LPO (Figure 6) in Indomethacin induced enterocolitis in rats.**

Values are expressed as mean ± SEM for 6 animals in each group, One Way ANOVA followed by Dunnett's Test, <sup>##</sup>P<0.01 when compared to Control, <sup>\*</sup>P< 0.05, <sup>\*\*</sup>P<0.01, <sup>\*\*\*</sup>P<0.001 when compared with Indomethacin.



**Figure No: 7 (A\_E) Histopathological images of normal and treated ileum**

### **Biochemical Assays**

Effect of Mandarin Oil treatment (200 mg/kg and 400 mg/kg) on Myeloperoxidase (MPO), Colonic glutathione (GSH), Colonic Lipid Peroxides (LPO) and Serum lactate dehydrogenase (LDH) concentration is shown in Table.1 Biochemical assay shows that administration of INDO leads to extensive colonic mucosal injury with increase in macroscopic score, serum LDH level and MPO activity, colonic LPO and decrease in feed intake and colonic GSH content along with intestinal dysmotility and damage to colonic structure. Pre-treatment with MO was effective in reversing these parameters. MO- I was found to be more effective than MO II. His-

tologically, damage was restored. The beneficial effects of MO are comparable to the standard drug SLZ (Figure 3-6).

### **Histopathology**

The Histopathological changes of the normal and treated rat ileum are shown below. Fig. 7A - Normal ileum of rat showed intact mucosa and epithelial surface; 7B - INDO treated inflamed ileum of rat showing submucosa with severe infiltration and edema; 7C, 7D - Ileum of MO treated (200 and 400 mg/kg respectively) rat showing recovered submucosal edema & few inflammatory cells associated with Indomethacin adminstra-

tion; 7E - Sulfasalazine treated rat showing recovered sub mucosal edema & severity of cell damage.

## DISCUSSION

The present study confirmed that treatment with the Mandarin oil, reduced the enterocolitis induced by TNBS and ethanol, as verified by macroscopic, histological and biochemical data.

Inflammatory bowel disease is characterized by the involvement of ROS in tissue damage. Increased granulocyte accumulation in inflammatory lesions of gut mucosa in patients with IBD has been shown. Those activated cells release a number of inflammatory mediators such as toxic oxygen metabolites, lysosome enzymes, and derivatives of arachidonic acid metabolism. It has been proposed that inflammation of mucosa causes impairment of the antioxidant defence mechanism, and makes tissue more susceptible to oxidative damage.<sup>19</sup>

In the present study, Mandarin oil has shown to be effective in indomethacin-induced enterocolitis in rats, which has protected the animals against experimentally induced disease because of its antioxidant and anti-inflammatory activities.<sup>6,12</sup> The Mandarin oil possess good antibacterial properties; therefore, it may be useful in combating intestinal or other pathogens, which play important role in the pathogenesis of IBD. It has antiulcer and antifungal actions, which might be useful in gastrointestinal disturbances & disturbed GI motility occurring in inflammatory bowel conditions. The mechanism of development of disease in Indomethacin-induced enterocolitis involves role of protective prostaglandins and intestinal pathogens.

## CONCLUSION

In conclusion, Mandarin oil may prove useful in the treatment of IBD. However, more detailed phytochemical studies are necessary to identify the active principles and exact mechanisms of action.

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